



Stefania Latrofa, M., Giannelli, A., Flaminia Persichetti, M., Grazia Pennisi, M., Solano-Gallego, L., Brianti, E., Parisi, A., Wall, R. L., Dantas-Torres, F., & Otranto, D. (2017). *Ixodes ventalloi*: morphological and molecular support for species integrity. *Parasitology Research*, 116(1), 251–258.
<https://doi.org/10.1007/s00436-016-5286-9>

Peer reviewed version

Link to published version (if available):
[10.1007/s00436-016-5286-9](https://doi.org/10.1007/s00436-016-5286-9)

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***Ixodes ventalloi*: morphological and molecular support for species integrity**

Maria Stefania Latrofa¹, Alessio Giannelli¹, Maria Flaminia Persichetti², Maria Grazia Pennisi³, Laia Solano-Gallego⁴, Emanuele Brianti³, Antonio Parisi⁵, Richard Wall⁶, Filipe Dantas-Torres^{1,7}, Domenico Otranto^{1*}

¹Department of Veterinary Medicine, University of Bari, 70010 Valenzano, Bari, Italy.

²Istituto Zooprofilattico Sperimentale della Sicilia, 90129 Palermo, Italy

³Department of Veterinary Sciences, University of Messina, Polo Universitario Annunziata, 98168 Messina, Italy

⁴Department of Animal Medicine and Surgery, Autonomous University of Barcelona, 08193 Bellaterra, Barcelona, Spain

⁵Istituto Zooprofilattico della Puglia e della Basilicata, 70017 Putignano, Bari, Italy

⁶University of Bristol, School of Biological Sciences and School of Veterinary Science, Bristol Life Sciences Building, BS8 1TQ Bristol, United Kingdom

⁷Aggeu Magalhães Research Centre, Oswaldo Cruz Foundation, Recife, Pernambuco, 50670-420, Brazil

*Corresponding author: D. Otranto

E-mail address: domenico.otranto@uniba.it

Tel +39 080 4679944/9839;

Fax +39 080 4679393

Abstract

Despite their medical and veterinary importance, some tick species are so poorly studied, that their role within pathogen vector transmission cycles is difficult to assess. The tick *Ixodes ventalloi* is one such species, and its biology and phylogenetic status remain an issue of debate. In the present study, specimens of adult *I. ventalloi* (n = 65 females; n = 31 males) infesting cats in the Lipari Island (Aeolian archipelago, Sicily, southern Italy) were characterized morphologically and molecularly, the latter based on mitochondrial 16S rRNA and cytochrome *c* oxidase subunit 1 (*cox1*) genes. In addition, within the *I. ventalloi* specimens examined, the genetic data and phylogenetic analyses for both mitochondrial genes suggest the existence of two distinct genogroups. The ecological and epidemiological significance of the genetic structure within the *I. ventalloi* endemic population remains to be determined. The results highlight the need for further analysis of this tick species, including whole mitochondrial genome sequencing and crossbreeding studies, which will be pivotal to complement features of its status as a vector of disease pathogens.

Keywords: *Ixodes ventalloi*, cats, morphology, 16S rRNA, *cox1*, phylogeny.

Introduction

Ticks of the genus *Ixodes* (Ixodida, Ixodidae) have a significant impact on public health in many parts of the world, due to their role as vectors of several pathogens, including viruses, bacteria and protozoa (de la Fuente et al. 2008; Dantas-Torres et al. 2012). Human and animal movement, associated with environmental changes have favoured the dispersal of some tick species and increased the risk of tick-borne diseases in previously free areas (Estrada-Peña 2008; Dantas-Torres 2015). However, many tick species remain so poorly studied that their role as pathogen vectors remains obscure.

The rabbit tick, *Ixodes ventalloi* is one such species, and knowledge of its natural history is limited. Nevertheless, it has been implicated as a potential vector of pathogens (e.g., *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Rickettsia monacensis*, *Bartonella clarridgeiae*, and Eyach virus) (Chastel et al. 1984; Santos-Silva et al. 2006; Marquez 2008; Hubalek and Rudolf 2012; Otranto et al. 2014; Pennisi et al. 2015). This tick primarily parasitizes the European rabbit (*Oryctolagus cuniculus*) at all developmental stages, but may occasionally be found on other hosts including cats, dogs, ground-dwelling birds and humans (Santos Dias and Santos-Reis 1989; Santos-Silva et al. 2011; Otranto et al. 2014; Pennisi et al. 2015).

Ixodes ventalloi has been reported in France, Tunisia, Morocco, Spain, Portugal, Italy, Germany and Great Britain (Chastel et al. 1984; Petney et al. 1996; Jameson and Medlock 2011; Santos-Silva et al. 2011; Estrada-Peña et al. 2014; Otranto et al. 2014; Mori et al. 2015; Pennisi et al. 2015). However, understanding its geographic distribution is complicated by the fact that it is difficult to differentiate from *Ixodes festai* (Gilot and Perez 1978; Petney et al. 1996). Thus, several records of these tick species have been regarded as doubtful (Contini et al. 2011; Guglielmone et al. 2014).

DNA sequencing and phylogenetic analysis employing mitochondrial (mt) DNA targets (e.g., 16S rRNA and 12S rRNA, *cytochrome c oxidase subunit 1*, *cox1*) have been demonstrated as valuable tools that can be used to complement the morphological identification of closely related tick species (Mangold et al. 1998; Murrell et al. 2000; Latrofa et al. 2013). However, no molecular data is available for *I. ventalloi*. The aim of this study, therefore, was to provide complementary molecular data to support the morphological description of this tick species.

Materials and Methods

Tick collection and morphological analysis

A total of 96 tick specimens (n = 65 females; n = 31 males) were collected, between March 2012 and April 2014, by a veterinary practitioner from outdoor owned and stray cats included in trap-neuter-release programs on Lipari Island (southern Italy) during the physical examination. Lipari is the largest of the Aeolian Islands (surface area of 37.6 km²) in the Tyrrhenian Sea off the northern coast of Sicily (38° 28' 3' N, 14° 57' 14' E), and has a Mediterranean climate.

Ticks were placed in vials containing 70% ethanol and were identified morphologically based on reference keys for Italian ixodids (Manilla 1998). Ticks were selected based on their preservation status and 10 males and 10 females, with no obvious indication of engorgement, were subjected to detailed morphometric examination using a stereomicroscope (Leica MS5) equipped with a digital camera (Leica DFC 425). Digital images were processed using the Leica Application Suite version 4.1 software (Leica Microsystems). The following features, considered taxonomically relevant for *Ixodes* spp. differentiation (Gilot and Pérez 1978; Contini et al. 2011), were carefully examined: shape of the palps, length of the coxae, presence/absence of cornua and auriculae, position of the genital aperture in females, shape of the adanal plates in males, and hypostomal dentition. Two females and two males of *I. ventalloi* were clarified with 10% KOH and lactophenol, and submitted as voucher specimens to the Laboratory of Parasitology and Parasitic Diseases of the University of Bari.

Molecular procedures and analyses

After morphological analysis, 92 specimens of *I. ventalloi* were subjected to genetic studies. For this, genomic DNA extraction was performed using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen GmbH, Hilden, Germany), in accordance with the manufacturer's instructions. Partial regions of the 16S rRNA and *cox1* genes (ca. ~300 and 500 bp,

respectively) were amplified according to Latrofa et al. (2013). PCR products were examined on 2% agarose gels stained with GelRed (VWR International PBI, Milano, Italy) and visualized on a GelLogic 100 gel documentation system (Kodak, New York, USA). The amplicons were purified and sequenced, in both directions using the same primers as for PCR, employing the Big Dye Terminator v.3.1 chemistry in a 3130 genetic analyzer (Applied Biosystems, California, USA). Sequences of the 16S rRNA and *cox1* genes were aligned using the ClustalW program (Larkin et al. 2007) and compared with those available in GenBank (BLAST – <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The percentage of nucleotide variation (Pairwise comparisons, Pwc %) amongst haplotypes of the 16S rRNA and *cox1* identified, was calculated using the Kimura 2 Parameter substitution model with Gamma Distributed (G) rates among sites (Kimura 1980), implemented in the MEGA6 software (Tamura et al. 2013). Consensus sequences from selected species, including *Ixodes ricinus*, *I. inopinatus*, *Ixodes nipponensis* and *Ixodes spinipalpis*, were generated from those available in GenBank for both genes using the BioEdit software (Hall 1999). Interspecific nucleotide differences of the consensus sequences were identified according to International Union of Pure and Applied Chemistry (IUPAC, <http://www.chem.qmul.ac.uk/iupac>) and the International Union of Biochemistry and Molecular Biology (IUBMB) codes. Pairwise comparisons of nucleotide differences (D) amongst *Ixodes* spp. consensus sequences were calculated using the formula $D = 1 - (M/L)$, where M is the number of alignment positions at which the two sequences have a base in common, and L is the total number of alignment positions over which the two sequences are compared (Chilton et al. 1995). For phylogenetic analyses, sequences of all available *Ixodes* spp. from GenBank were also included. The phylogenetic relationships were inferred by Maximum Likelihood (ML) (Kimura 1980) analysis, conducted using the General Time Reversible (GTR) model using MEGA6 software for both genes (Tamura et al. 2013). To estimate the molecular evolutionary relationship and

the divergence times the RelTime method (Tamura et al. 2012) was applied and the divergence times for all branching points in the topology were calculated using the ML method based on the Kimura 2 parameter model (Kimura 1980) using MEGA6 software (Tamura et al. 2013). For each gene, homologous sequences from *Rhipicephalus sanguineus* s.l. (Accession numbers: 16S rRNA KC243835; *cox1* KC243875) were used as outgroup.

Results

Morphological identification

All ticks were identified as *I. ventalloi* (Fig. 1) and a description of salient morphological features of both females and males is provided below.

Ixodes ventalloi female: pointed and strongly curved auriculae (Fig. 2); coxae I with anteroposterior spine very slender, long and curved, forming an angle with the inner edge of the coxa; coxae II and III with sharpened spur but distinct internal spurs and short external spurs (Fig. 3a); tarsi I gradually tapering (Fig. 3b); the genital aperture arched in shape and positioned between coxae IV; spiracular plates oval. Palps long and rounded on the apical part, with sutures clearly observed between articles II and III. Hypostome long, featured by distinct hypostomal dentition with three rows of 2/2 on the basis and eight rows of 3/3 on the apex.

Ixodes ventalloi male: bristles evident on the lower part of the shield, together with punctations; scapular grooves short, scapulae rounded and rectangular basis capituli, with robust cornuae (Fig. 1b); adanal plates wider in the anterior part and anal shield lacking setae;

capitulum with short, broad palps, hypostome with eight pairs of teeth; auriculae strongly reduced and coxae I with distinct internal and external spurs (Fig. 4).

Molecular and phylogenetic analyses

The BLASTn analyses of all *I. ventalloi* sequences (n = 92 16S rRNA, n = 77 *cox1*) revealed the highest nucleotide identity (range, 88-91%) with those of *I. ricinus*, *I. spinipalpis*, *I. nipponensis* and *Ixodes persulcatus* from GenBank (i.e., 16S rRNA: JN248424, L34297, AF549856 and *cox1*: JX288764, JX141654, AB231671). Eight 16S rRNA (aligned over 279 sites, 78.2% AT) and 16 *cox1* (aligned over 455 sites, 65.7% AT) haplotypes were identified within the *I. ventalloi* populations (Tables 1 and 2). For *cox1* except that for one nucleotide substitution (Tyr-Asp) all the others were synonymous. Haplotype I was identified in 64 (69.6%) and 27 (35%) tick specimens for 16S rRNA and *cox1*, followed by haplotype III (n = 18; 19.6%), II (n = 24; 31.2%) and others (Table 2).

The overall intraspecific variation calculated for both genes amongst all haplotypes was up to 5%, revealing the existence of two distinct genogroups (i.e., named as A and B) amongst all *I. ventalloi* examined. The mean genetic distance within genogroups A and B was up to 1.3% and 2%, respectively (Tables 1 and 2). The level of inter- and intra-specific pairwise distance of other *Ixodes* spp. ticks was up to 20.4% and 3.9%, respectively (Tables 3 and 4). The association of each tick specimen to a specific genogroup was confirmed by both genes regardless of sex (Table 2).

The ML trees inferred from mitochondrial 16S rRNA and *cox1* sequences revealed a similar population genetic structure to that identified by the pairwise nucleotide analyses, displaying two monophyletic sister clades with high bootstrap values (i.e., 98-99%) at their main branches, distinct from other *Ixodes* spp. Timetree analysis revealed that relative time differences between haplotypes within a genogroup are lower (node time values 0.00-0.01)

than between genogroups (node time values 0.01-0.02) (Fig. 5). Measurements and comparison of key characteristics of male and female ticks, of each genogroup, did not reveal significant differences among specimens (t -test, $p < 0.05$; Table 5).

All representative haplotypes of *I. ventalloi* obtained were deposited in GenBank (16S rRNA: KU178956-KU178963; *cox1*: KU178964-KU178979).

Discussion

This study provides new molecular data regarding the tick *I. ventalloi*, which is a poorly studied species. The initial formal description of *I. ventalloi* (Gil Collado 1936) was performed using specimens obtained from an owl, *Athena noctua*. Nevertheless, this tick largely parasitizes rabbits at every development stage. Following its initial description, *I. ventalloi* was synonymised with *Ixodes thompsoni*, and then with *I. festai*, based on the description of a male and re-description of female tick specimens collected from rabbits (Arthur 1963, 1965). Subsequently, Bailly-Choumara et al. (1974) suggested that *I. festai* sensu Arthur was a synonym of *I. ventalloi*. However, their status as separate species was then reasserted by Gilot and Perez (1978), who provided clear morphological features for the identification of *I. ventalloi*. All tick specimens analysed in the present study were characterized by the robust, pointed and arcuated internal spur of coxa I and by the short, wide profile of tarsus I, with an untapered end. In addition, they bear long auriculae, curved towards the animal symmetry axis (Gilot and Perez 1978; Contini et al. 2011). While morphological features indicate that all *I. ventalloi* specimens display the same phenotype, molecular analyses highlight the existence of a significant genetic structure within the tick population examined. Indeed, the overall intraspecific nucleotide variation found for both mitochondrial genes, revealed the occurrence of two genogroups, named A and B. The nucleotide variation between these genogroups was lower than the interspecific genetic

distance found in other *Ixodes* spp. (i.e., up to 20.4% for 16S rRNA and 13.2% for *cox1*). Matching the high degree of nucleotide divergence of mitochondrial markers, the corresponding ML phylograms included two robust clades for *I. ventalloi* (bootstrap value up to 99%). The Timetrees analysis reported a low relative time value (0.01-0.02), which suggests a recent separation between the two *I. ventalloi* genogroups.

The genetic heterogeneity detected for *I. ventalloi* could be related to the high mutation rate of mitochondrial DNA (Avice 1994). Nonetheless none of the nucleotide variation in *cox1* haplotypes resulted in amino acid changes, except one which did not cause any functional change.

The presence of high genetic variability within a tick species has been previously documented, for example in *I. scapularis* (16S rRNA, Krakowetz et al. 2011) and *Rhipicephalus (Boophilus) microplus* (Burger et al. 2014). The reasons for the presence of two clades within the *I. ventalloi* population is unknown, especially given that, all tick specimens have been collected from cats, irrespective of their behaviours, living in a limited geographical area. However, it is possible to hypothesize that tick specimens, with both genotype, originally came from different geographical regions and which were accidentally found in the same studied area, as has been postulated for *I. scapularis* (Krakowetz et al. 2011). A large recent expansion of *I. ventalloi* populations has been also described in Portugal (Santos-Silva et al. 2011). Unfortunately, no molecular data is available for *I. ventalloi* specimens in any of the previous reports (Chastel et al. 1984; Jameson and Medlock 2011; Santos-Silva et al. 2011; Estrada-Peña et al. 2014). Thus, it is not possible to identify the haplotypes occurring in other parts of Europe or to establish the relationship between its genogroup and the positivity to different pathogens.

The same haplotype I of genogroup A of *I. ventalloi* was here identified for a tick specimen (Accession number: 16S rRNA KU178956; *cox1* KU178964) collected on humans and

scored positive for *R. helvetica* (Otranto et al. 2014), therefore raising the question about the potential role of this tick species as a vector of zoonotic pathogens (Gilot and Marjolet 1982). Although the affiliation of *I. ventalloi* to lagomorphs makes its distribution patchy (Manilla, 1998), the transmission of this tick species from the rabbit to domestic or wild cats may occur, especially in areas like the Aeolian Islands, where a wide population of wild lagomorphs and of domestic cats coexist (Pennisi et al. 2015). Whether the presence of these genogroups of *I. ventalloi* is related to the existence of two distinct populations infesting rabbits and cats is yet to be determined.

In conclusion, based on the data presented here and in the absence of morphological differences, the molecular analysis is the only method that can reliably discriminate between the two genogroups of *I. ventalloi*. The results highlight the need for further analyses of this tick species, including whole mitochondrial genome sequencing and crossbreeding studies, which will be pivotal to complement studies of its status as a vector of disease pathogens.

Acknowledgments

The authors would like to thank Dr. Viviana Domenica Tarallo (University of Bari) for the *I. ventalloi* drawing in Figure 2.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

Fig. 1 Female (a) and male (b) *Ixodes ventalloi habitus*, ventral view (scale bar = 500 μm)

Fig. 2 Picture and drawing of *I. ventalloi* female (scale bar = 200 μm). The arrow highlights the auricula, which appears as a long claw, with a sharp tip and accentuated curvature, inclined towards the tick symmetry axis

Fig. 3 Spur on coxa I and tarsus I of *I. ventalloi* (a, b) (scale bar = 200 μm). In a, the spur on coxa I is slender, long and curved forming an angle with the inner edge of coxa (arrow); in b, tarsus I is featured by a short and wide end, with a wide apical slope (arrow)

Fig. 4 *Ixodes ventalloi* male: base of the capitulum (a, scale bar = 100 μm), capitulum and hypostomal dentation (b, scale bar = 200 μm); coxae (c, scale bar = 200 μm) and stigmata (d, scale bar = 100 μm)

Fig. 5 Phylogenetic trees based on mitochondrial 16S rRNA (a) and *cox1* (b) sequence data for *Ixodes ventalloi* along with those of other *Ixodes* species available in the GenBank. The bootstraps (>50 %) and relative time value (i.e., relative number of substitutions per site) for each internal node are indicated. The trees were generated using the RelTime method and the divergence times were calculated using the Maximum Likelihood method based on the Kimura 2-parameter model